Counting the calories to stay in the groove

High-sensitivity microcalorimetry is beginning to make an impact on the determination of thermodynamic parameters associated with protein–DNA interactions and the understanding of the relationship of these data to structural details of complex formation.

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The fundamental biological control systems mediated by equilibrium protein–DNA interactions can be defined by the interdependence of thermodynamic properties (measured in calories) with structural detail. Thus, determination of the physico-chemical parameters and the relationship of these to the structural consequences of changing the system from the free state to the bound state is the key to comprehending how interactions occur. The development of high-sensitivity microcalorimetric instrumentation makes the direct measurement of thermodynamic parameters associated with protein–DNA interactions accessible. Coupled with the availability of high-resolution structures of proteins, DNA molecules and protein–DNA complexes we are in a good position to begin to understand the rules governing these interactions.

Calorimetric method

Unlike all other techniques used to determine thermodynamic parameters, calorimetry, as the name suggests, uses the direct measurement of the heat of interaction (i.e. the enthalpy, ΔH) to probe the extent of reaction. Enthalpy can be measured with great accuracy (typically the minimum detectable heat pulse is 0.2 μcal). In the other techniques, such as those measuring the change in a property of the interacting species using spectroscopic probes (e.g. fluorescence and UV radiation), the enthalpy has to be calculated indirectly from the temperature dependence of the equilibrium binding constant, K_B, using the van't Hoff relationship.

The most frequently used calorimetric technique for exploring protein–DNA interactions is isothermal titration calorimetry (ITC) [1,2], although pulsed-flow methods have been employed for enthalpy measurement [3] and differential scanning calorimetry can be used for determination of tight-binding events [4]. For a review of calorimetric techniques and the application of calorimetric data, see [5] and [6] respectively.

In an ITC experiment the enthalpy of binding is measured from a small reaction cell (with approximate volume 0.5–1.5 ml). Two identical cells (one a reference) are kept at constant reaction temperature by heating. Aliquots of a solution of one of the interacting molecules, say the protein, are injected into the other, the DNA, in the reaction cell. An endothermic or exothermic enthalpy will be associated with this interaction. Thus, to maintain a constant temperature the amount of heat applied to the reaction cell has to be either increased or decreased respectively. It is this change in heat requirement of the reaction cell to maintain isothermal conditions (with respect to the reference cell) that is measured. The concentrations of the protein and the DNA are such that, over the course of the experiment, the available binding sites on the DNA gradually become saturated and so the corresponding enthalpy per injection is reduced. Ideally, by the last few injections the only heat change observed is due to the dilution effect of protein into the solution in the cell, as no net additional binding occurs. The heats per injection are calculated and because the concentrations of the protein and DNA are known the molar enthalpy for the binding interaction can be determined. For a simple interaction, if enthalpy is plotted against injection number (or molar ratio) a sigmoidal titration curve is obtained as the binding sites become saturated (see, for example, Fig. 1). The profile of this curve is dictated by K_B. Fitting this curve to a simple algorithm enables the K_B and the ΔH to be determined directly. Having established these parameters for a given interaction, full thermodynamic characterization can be obtained in one rapid experiment (typically taking 1–2 h) based on the relationship:

$$-RT\cdot\ln K_B=\Delta H-T\Delta S^\circ=\Delta G^\circ$$

where R is the gas constant, T is the absolute temperature, ΔS° is the standard state entropy change and ΔG° is the standard state free energy change for the interaction. These thermodynamic quantities are affected by the variables of solution pH, ionic strength, concentration of interactants, temperature and pressure [7].

If titrations are performed over a range of temperatures the change in heat capacity at constant pressure, ΔCp, for the interaction can be determined, because

$$\Delta Cp=(\Delta H_{T2}-\Delta H_{T1})/(T2-T1)$$

where T1 and T2 are two different experimental temperatures. ΔCp is related to entropy by the following equation:

$$\Delta Cp=(\Delta S^\circ_{T2}-\Delta S^\circ_{T1})/\ln(T2/T1)$$

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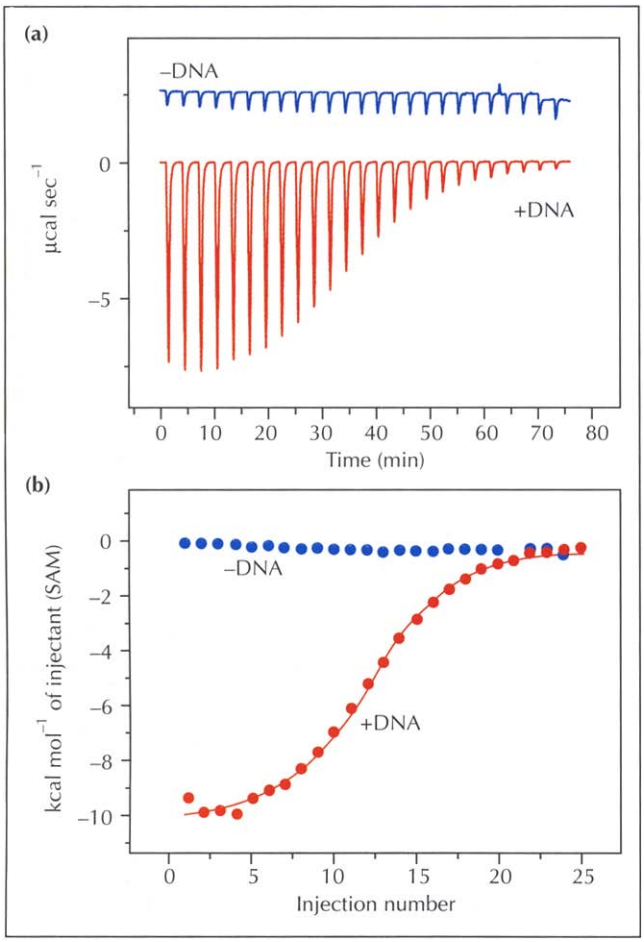

Fig. 1. Isothermal titration data at 25°C for 25 injections of the corepressor, *S*-adenosylmethionine (SAM) (2.2 mM), in 10 µl aliquots with the methionine repressor, MetJ (85 µM) in the presence or absence of the operator (met box) DNA (150 µM) [9]. **(a)** Raw titration data showing the exothermic response for each injection. **(b)** Normalized heat of interaction data obtained by integration of raw data from (a), corrected for heat of dilution. The solid line is the result of a non-linear least-squares fit for non-cooperative binding of SAM to MetJ–DNA with $K_B=9.3\times10^4$ M^{-1} and $\Delta H=-10.5$ kcal mol^{-1}; assuming two SAM-binding sites per repressor. (Figure courtesy of A Cooper.)

Microcalorimetry, and more specifically ITC, can be applied to the investigation of several aspects of protein–DNA interactions as detailed below.

The study of complex systems

In addition to the thermodynamic characterization described above, ITC also enables the determination of the stoichiometry for a given interaction based on the molar ratio of the system at the equivalence point of the titration. This value has to be assumed in many of the other techniques used to obtain thermodynamic parameters.

Thus, ITC can be valuable in extracting data from more complex interactions (i.e. those involving more than one type of interaction). A good example of this was observed in the interaction of *trp* repressor with a 20 base-pair oligonucleotide designed to represent the *trp* operator sequence [8] (Fig. 2a). In this interaction the homodimeric repressor protein recognizes the sites shown on the palindromic duplex forming a tight primary interaction. However, there was a second potential interaction site for the protein on the chosen oligonucleotide. This corresponded to a half-site secondary interaction, which would be expected to be weaker than the primary interaction. ITC data gave non-sigmoidal titration curves — that obtained at 13°C is shown in Figure 2b. These data clearly revealed the presence of two interactions. At 13°C an initial exothermic tight-binding event occurred with a stoichiometry of one repressor dimer to one DNA 20-mer ($K_B\cong1\times10^9$ M^{-1}; $\Delta H=-6.5$ kcal mol^{-1}). As this tight-binding site became saturated, binding began at the weaker secondary half-site, which at this temperature was an endothermic event ($K_B\cong2.2\times10^5$ M^{-1}; $\Delta H=+10.94$ kcal mol^{-1}). This was finally saturated as indicated by no further heat of binding (after subtraction of the heats of dilution of buffer into DNA and protein into buffer). This combined binding would not have been discernible by spectroscopic techniques and hence could lead to erroneous results.

The control-feedback requirements of physiologically relevant interactions involving proteins and oligonucleotides are, for the most part, complex and multimolecular. For example, gene regulatory systems often involve the linkage between reversible protein oligomerization and DNA binding, and/or require the presence of a cofactor. Thus, understanding these interactions from a thermodynamic point of view requires a reductionist approach. This is best achieved by disassembling the complex system into binary and ternary interactions. In the case of the MetJ repressor binding to its met box operator DNA, several equilibria are involved. Individual experiments of corepressor (*S*-adenosylmethionine, SAM) binding to repressor protein, aporepressor binding to DNA, holorepressor binding to DNA and SAM binding to the protein–DNA complex were performed to characterize the system (the presence of corepressor increases the binding affinity of the protein to DNA by 2 kcal mol^{-1}) [9,10]. The results of these studies were all incorporated into a global binding model which enabled estimates to be made of the cooperative effects of the binding of repressor to tandem met boxes [10]. In the *trp* repressor–operator system, separate binding studies of the tryptophan cofactor with the repressor (performed by ITC) and of the holorepressor with DNA indicated that the presence of the tryptophan cofactor significantly affected the protein–DNA interaction [11].

Thermodynamic investigation of specific and non-specific interactions

A protein is able to select a specific binding site from DNA, which presents many potential sites all sharing a high degree of structural similarity. Hence, establishing the thermodynamic cost of protein–DNA recognition poses an intriguing problem [12–14]. Proteins can interact with DNA in a non-sequence-specific manner. Indeed, these non-specific interactions may play a

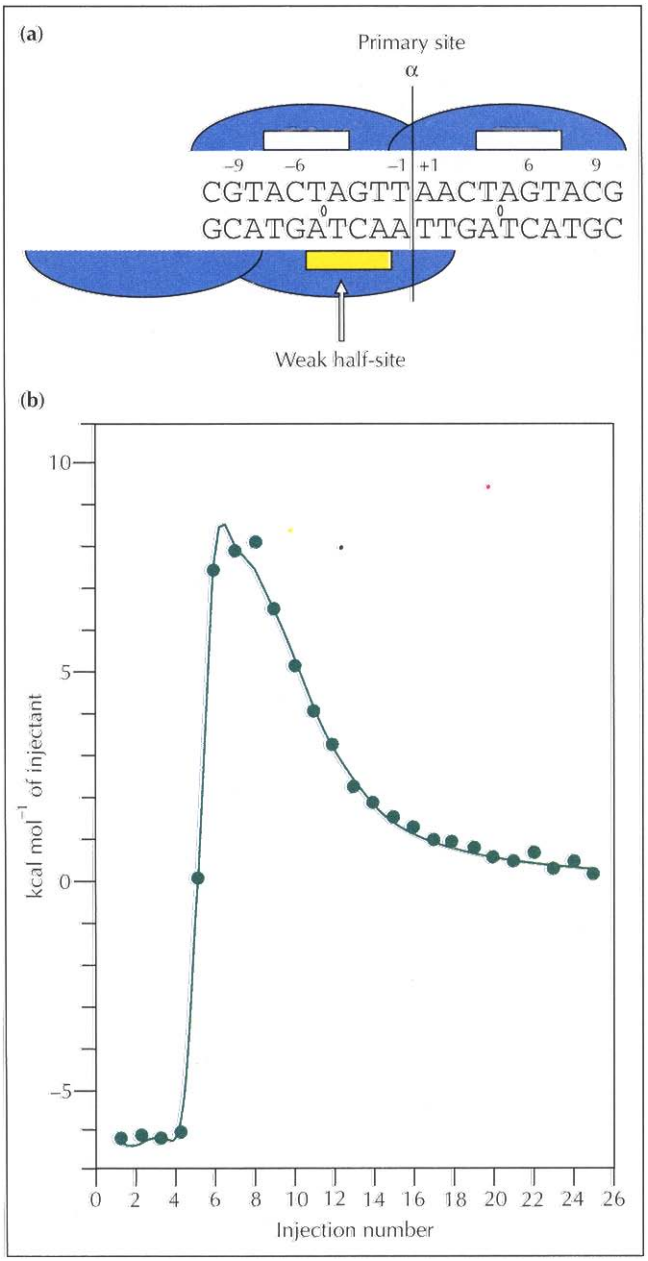

Fig. 2. (a) Schematic diagram of the *trp* repressor binding to the 20 base-pair *trp* operator DNA showing the potential primary and secondary binding modes. α indicates the palindrome's dyad axis. **(b)** ITC data for the 25 × 10 μl injections of *trp* repressor (0.615 mM) into *trp* operator DNA (42.1 μM) at 13°C [8]. The solid line is the result of a non-linear least-squares fit for two independent binding events.

physiological role in localization of protein to its cognate site by a one-dimensional search. There are distinct thermodynamic consequences pertaining to formation of specific *versus* non-specific interactions.

Under typical conditions of ionic strength, specific protein–DNA interactions are characterized by high affinities. In some cases, the useful range of measurement of current ITC instrumentation (10^3–10^9 M^{-1}) is not sufficient for direct K_B determination, although ITC can be applied to the determination of high-affinity cooperative interactions by indirect methods [15]. These tight-binding events translate into large negative $\Delta G°$ values, which are essentially temperature-independent. Interactions of proteins with non-specific DNA are significantly weaker (at 35°C *trp* repressor dimer $\Delta G°_{(specific)}$=−11.8 kcal mol^{-1}, $\Delta G°_{(non\text{-}specific)}$=−6.8 kcal mol^{-1} [8]; at 25°C MetJ dimer $\Delta G°_{(specific)}$=−7.7 kcal mol^{-1}, $\Delta G°_{(non\text{-}specific)}$=−5.7 kcal mol^{-1} [10]). Typically, the enthalpy of non-specific protein–DNA interactions is low (at 25°C MetJ–operator DNA $\Delta H \cong -2$ kcal mol^{-1} [9,10]). This indicates that the entropic term is dominant in these interactions. This favourable entropic effect could be accounted for by removal of interacting water molecules into bulk solvent from the binding site of protein and DNA. The non-specific association has been hypothesized to be essentially an electrostatic effect, with specific hydrogen bonds and van der Waals contacts lacking.

Specific interactions also appear to be accompanied by large negative changes in heat capacity, for example: *trp* repressor dimer–operator ΔCp=−0.95 kcal mol^{-1} K^{-1} [8]; MetJ dimer–operator ΔCp=−0.58 kcal mol^{-1} K^{-1} [10] (it should be noted that a smaller value of ΔCp has been reported [9], and the reason for this discrepancy is not clear); and Cro dimer–operator ΔCp=−0.36 kcal mol^{-1} K^{-1} [16]. The consequence of this large negative ΔCp is that the enthalpic ΔH and entropic $T\Delta S°$ contributions to $\Delta G°$ show a linear variation with temperature. They act in a compensatory way, resulting in near temperature independence of $\Delta G°$, as mentioned above. The values of entropy and enthalpy generally change sign in the physiological temperature range. Non-specific interactions appear to be characterized by low or zero ΔCp [7,16]. This lack of dependence of ΔH on temperature is commensurate with a weak complex held together by electrostatic forces as mentioned above. Microcalorimetry provides an ideal method for determining ΔCp because the enthalpy is measured directly.

Relationship of thermodynamics to structural changes

There is now great interest in relating the thermodynamic and structural determinants of protein–DNA interactions. Biotechnology is the main driving force behind these efforts to find predictive algorithms that will facilitate drug design. On the basis of data from protein folding/unfolding equilibria, and transference of aliphatic hydrocarbons to aqueous solvents, a general relationship between the ΔCp and the burial of surface area (more importantly hydrophobic surface area) from exposure to solvent has been established [17,18]. Because it is based on the same principle, this relationship should hold for molecular interactions whereby surface area is buried in the interface of the associated molecules. To explore this relationship, high-resolution structural data of the interacting molecules and the complex are required to complement thermodynamic data. This structural information for protein–DNA interactions is now becoming available. The magnitude of the predicted ΔCp derived from the limited structural information of surface area burial in protein–DNA complexes was

significantly smaller than that observed [17]. Assuming that surface area burial is solely responsible, for ΔCp this discrepancy can be dealt with by invoking conformational change within one or both of the interacting molecules resulting in additional buried surface area on binding [18]. This 'induced fit' model is attractive when one considers the severe conformational changes observed in some protein–DNA complexes.

In the binding of *trp* repressor with *trp* operator, the predicted ΔCp from the empirical surface area burial relationship (based on crystal structures of both interactants and the complex determined at better than 2 Å resolution) was approximately half that obtained from the ITC experiment, and no major conformational change was apparent from the X-ray crystallographic studies [8]. Thus, other contributions to ΔCp deserve further investigation. The most prominent of these is the effect on interaction of restriction of vibrational modes of molecules [19]. The interface between *trp* repressor and operator shows a preponderance of water molecules (Fig. 3). Some of these water molecules make specific contacts, and loss of these contacts significantly affects ΔG° [20,21]. It is hypothesized that these water molecules could make a contribution to the ΔCp as their vibrational modes are severely restricted compared with water in bulk solvent.

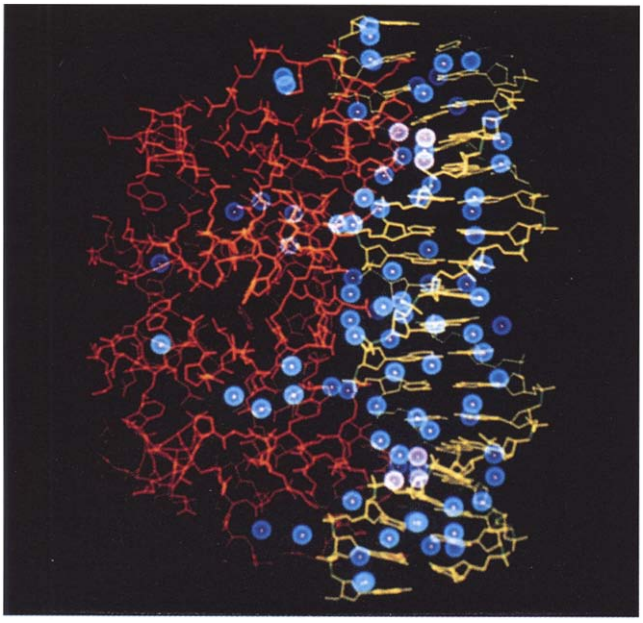

Fig. 3. Structure of the dimeric *trp* repressor (orange) bound to the *trp* operator DNA (yellow) depicting the water molecules that are common to all four of the independently determined crystallographic representations of the half-repressor–operator complex (1.9 Å). Water molecules are deemed to be at equivalent sites when they have the same nearest neighbouring atoms and the root mean square deviation of the distances to these four neighbouring atoms is less than 1 Å. The pink sites, three in each half-site, are water molecules found to be fixed in the free repressor and the DNA structure. (Figure courtesy of P Sigler.)

The 'dynamic' model has been proposed to explain the thermodynamic/structural relationship for the MetJ–DNA interaction [8]. This is based on the observation that on addition of the corepressor SAM to a MetJ–DNA complex no gross conformational changes are detected in the protein or DNA crystal structures. A significant 'shrinking' of the repressor molecule is observed resulting in a tightening of structure, restriction of vibrational modes (observed in B-factor changes) and an increased negative enthalpy being the dominant contribution to ΔG° on going from the free to the bound state.

Conclusion

We are now at a very exciting stage in the investigation of protein–DNA interactions. With the ability to measure accurately the thermodynamics of interaction and the increased availability of structural information we are in a position to discover what drives these interactions. Calorimetry is becoming a major tool in the investigation of protein–DNA interactions, providing perhaps the most valuable source of thermodynamic data. Ultimately, characterization of the thermodynamic/structural determinants will not only provide us with a better understanding of this fundamental heteromolecular interaction of life, but will lead also to predictive methods to allow thermodynamic quantification from structural knowledge with implications for pharmaceutical intervention.

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